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Generation of a Spacing Pattern: The Role of *TRIPTYCHON* in Trichome Patterning in Arabidopsis

Arp Schnittger, Ulrike Folkers, Birgit Schwab, Gerd Jürgens, and Martin Hülskamp[1]

Entwicklungsgenetik, Zentrum für Molekularbiologie der Pflanzen, Universität Tübingen, Auf der Morgenstelle 1, D-72076 Tübingen, Germany

Trichomes in Arabidopsis are single-celled hairs that exhibit a regular spacing pattern. Here, the role of *TRIPTYCHON* (*TRY*) in the generation of this spacing pattern is studied. By using genetic mosaics, we demonstrate that the formation of trichome clusters in *try* mutants is not correlated with cell lineage, indicating that *TRY* is required to single out trichome cells in a process involving cellular interactions. The genetic interactions of *TRY*, *GLABRA1* (*GL1*), and *TRANSPARENT TESTA GLABRA* (*TTG*) in trichome patterning are assessed by determining the cluster frequency in various genetic combinations. It is shown that *TRY* acts as a negative regulator of *GL1*- and *TTG*-dependent pathways. Furthermore, it is demonstrated that trichome initiation in *ttg-1*, a strong *ttg* allele, is rescued almost to wild-type levels in a *try* background in which *GL1* is expressed under the control of the cauliflower mosaic virus 35S promoter, indicating that *TTG* acts upstream of *GL1* and *TRY*. These findings are incorporated into a model to explain the generation of a trichome spacing pattern from a homogeneous population of epidermal cells.

INTRODUCTION

During the development of higher organisms, the spatial arrangement of differentiated cells is generated by a process called pattern formation. In most of the cases described to date, pattern formation integrates spatial information that is already present. In some cases, a pattern is generated de novo, meaning that initially all cells have the same competence and that during development, individual cells are singled out to acquire a different cell fate. De novo pattern formation could involve a stereotyped cell division program that has the intrinsic property of segregating different cell fates (cell lineage scenario). Alternatively, a pattern could arise from equivalent cells competing with each other by intercellular interactions to produce asymmetries that eventually result in the selection of individual cells (cell–cell interaction scenario).

Pattern formation in the leaf epidermis of the flowering plant Arabidopsis is a model system suitable for studying de novo patterning (Sylvester et al., 1996; Larkin et al., 1997; Marks, 1997; Hülskamp and Schnittger, 1998; Hülskamp et al., 1999). Two cell types, stomata and trichomes, show a characteristic spacing pattern: they are regularly distributed on the epidermal surface with a specific distance between the respective cell types. In both cases, pattern formation takes place in a two-dimensional field of cells. Stomatal patterning is mainly established by a series of highly oriented asymmetric cell divisions placing the two guard cells in the center of the future stomatal complex (Yang and Sack, 1995; Serna and Fenoll, 1997). As a result, guard cells are always surrounded by epidermal pavement cells and, thus, are separated from other stomatal cells. Although several observations suggest that lateral inhibition of stomatal cell fate can occur, the observed stereotyped division pattern is in principle sufficient to account for the observed spacing pattern (reviewed in Larkin et al., 1997).

Trichomes are large single cells that are surrounded by approximately eight accessory cells of characteristic morphology but of unknown function (Larkin et al., 1997; Marks, 1997; Hülskamp and Schnittger, 1998; Hülskamp et al., 1999). In contrast to stomatal patterning, the trichome–accessory cell complex does not appear to be derived from a stereotyped division pattern. This was shown by Larkin and co-workers, who demonstrated that early-induced β-glucuronidase sectors may divide the trichome–accessory cell complex, indicating that trichomes and accessory cells are not closely related (Larkin et al., 1996). This finding suggests that cells in immediate contact with a trichome cell become recruited as accessory cells after the trichome pattern has been established. This rules out a cell lineage scenario whereby a stereotypic series of cell divisions forms a complex in which the trichome cell becomes completely surrounded by its precursor cells. However, other cell lineage

[1] To whom correspondence should be addressed. E-mail martin.huelskamp@uni-tuebingen.de; fax 49-7071-29-57-97.

scenarios or trichome patterning based on cell–cell interactions are still consistent with these findings.

To date, four genes have been identified as playing a role in trichome initiation and/or trichome patterning. These include *REDUCED TRICHOME NUMBER* (*RTN*), *GLABRA1* (*GL1*), *TRANSPARENT TESTA GLABRA* (*TTG*), and *TRIPTYCHON* (*TRY*) (Koornneef et al., 1982; Hülskamp et al., 1994; Larkin et al., 1994, 1996). Whereas *RTN* appears to control the "developmental time window" during which trichome initiation occurs, *GL1*, *TTG*, and *TRY* are thought to be involved in the generation of the spacing pattern. All three share, either alone or in certain genetic combinations, a characteristic patterning defect: the formation of trichome clusters rather than individual trichomes. Mutations in the *TRY* gene result in the formation of trichome clusters containing up to four trichomes, suggesting that *TRY* functions to inhibit trichome cell fate in neighboring cells (Hülskamp et al., 1994). In addition, trichomes in *try* mutants show an increased number of endoreduplication cycles (Hülskamp et al., 1994).

By contrast, strong *gl1* and *ttg* alleles lack virtually all trichomes, suggesting that *GL1* and *TTG* act as positive regulators of trichome development. Genetic evidence suggests that both genes are involved early in trichome patterning. Several weak *ttg* alleles are known to produce trichomes that are often found in clusters (Larkin et al., 1994). *GL1* appears to function differently because none of the weak *gl1* alleles described to date exhibits trichome clusters (Esch et al., 1994; this study). However, a role of *GL1* in trichome patterning was suggested by Larkin et al. (1994) when they analyzed *GL1*-overexpressing plants that were heterozygous for a strong *ttg* allele. Whereas plants overexpressing *GL1* did not exhibit trichome clusters, the additional reduction of *TTG* dosage resulted in a large number of clustered trichomes, suggesting that the relative dosage of both genes is important during pattern formation (Larkin et al., 1994).

GL1 encodes a protein with sequence similarity to myb-related transcription factors (Oppenheimer et al., 1991). *GL1* expression is initially found uniformly in all epidermal cells at the base of young leaves and accumulates in developing trichome cells to high levels while transcription ceases in surrounding epidermal cells (Larkin et al., 1993). The analysis of genetic mosaic patterns suggests that *GL1* acts locally because *gl1* mutant sectors are not rescued by neighboring wild-type tissues (Hülskamp et al., 1994). Most of the molecular analyses directed toward the function of the *TTG* gene have focused on the *R* gene, because the overexpression of the heterologous *R* gene from maize can rescue all aspects of the phenotype of *ttg* mutants (Lloyd et al., 1992). The product of the maize *R* gene shows sequence similarity to myc-related transcription factors and, like *TTG* in Arabidopsis, is required for the regulation of anthocyanin biosynthesis (Ludwig et al., 1989). The recent cloning of the *TTG* gene has shown that TTG does not encode an *R* gene homolog (Walker et al., 1999), suggesting that the *R* gene acts downstream of *TTG* if it has a functional homolog in Arabidopsis. The *TRY* gene has not been cloned.

In this study, we used genetic mosaics to show that trichome clusters in *try* mutants are not derived from clonally related cells, suggesting that *TRY* functions to single out trichome cells in a process that does not involve cell lineage. To determine the role of *TRY*, *GL1*, and *TTG* in trichome patterning, we studied the genetic interactions among the three genes in various double and triple mutant combinations. Our results suggest a genetic model for de novo trichome patterning in Arabidopsis.

RESULTS

Formation of Trichome Clusters in *try* Mutants Does Not Involve Cell Lineage

The phenotype of *try* mutants is characterized by the development of up to four trichomes in the place of a single trichome. This phenotype is in keeping with *TRY* being involved in either of two patterning mechanisms: cell lineage or cell–cell interactions. In a cell lineage scenario, *TRY* would be involved in the control of cell fate decisions during cell divisions. Hence, clustered trichomes would have the same clonal origin, because they were derived from one common ancestor cell. In a cell–cell interaction scenario, *TRY* would function to prevent neighboring cells from adopting the fate of trichome cells. In this scenario, clustered trichomes would develop from cells that may or may not be clonally related.

To determine the clonal relationship of trichomes in *try* clusters, we induced somatic *stichel* (*sti*) clones by mutagenizing the F_1 seed of *sti*/+ *try*/*try* plants with ethyl methanesulfonate. If a new mutation is induced in the wild-type allele, the corresponding cell is a homozygous mutant for *sti*; if this cell contributes to mature vegetative epidermal tissues, it will give rise to a mutant *sti* sector. Epidermal *sti* sectors can easily be recognized as fields of unbranched trichomes on mature leaves (Figure 1A) (Schnittger et al., 1996). Borders of mutant *sti* sectors were inspected for *try* clusters through which a sector border passed (Figures 1B and 1C). Of 173 *sti* sectors, we identified three *try* clusters, each consisting of one unbranched trichome and two branched trichomes (Figure 1B). This finding argues against the cell lineage scenario and suggests that *TRY* is involved in the regulation of cell fate decisions mediated by intercellular interactions.

Criteria Used to Study the Role of *TRY*, *TTG*, and *GL1* in Trichome Patterning

Patterning mechanisms not involving cell lineage either use spatial information already provided by other cells or generate positional information de novo. Because trichome pat-

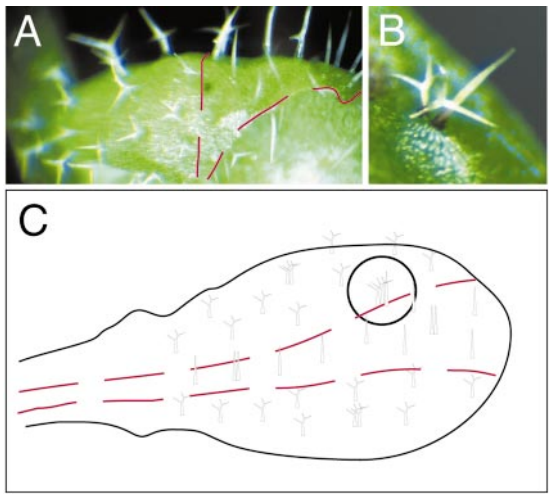

Figure 1. Sector Analysis of Cluster Formation in *try* Mutants.

(A) Overview of a mature rosette leaf with a *sti* sector in a *try* mutant background. Red lines indicate the boundary of the *sti* sector.
(B) *try* cluster consisting of two branched and one unbranched *sti* mutant trichomes.
(C) Schematic representation of a *sti* sector in a *try* mutant background. The *sti* sector encompasses part of the leaf with the sector border passing through a *try* cluster (circled).

terning does not appear to be correlated with the spatial distribution of other epidermal (e.g., stomata) or subepidermal cell types (e.g., vascular cells), it is likely that trichomes are initiated solely with reference to each other.

To study the role of *TRY*, *TTG*, and *GL1* in trichome patterning, two different criteria can be used to evaluate mutant phenotypes: (1) the frequency of clustered trichomes and (2) the distance between trichomes. The latter criterion, however, is ambiguous because changes in the distance between trichomes in mature leaves do not necessarily reflect alterations in the initial trichome patterning in different mutants. For example, mutants affecting the division rate of leaf epidermal cells would alter trichome density after trichome initiation has already taken place. In this study, we examined the trichome cluster frequency under the assumption that it reflects quantitative changes in the interactions of the respective genes. The cluster frequency is the percentage of trichome clusters per total number of trichome initiation sites. Trichome initiation sites are leaf positions with one trichome or a cluster of trichomes. To exclude secondary patterning events (for example, the transformation of accessory cells into trichomes, see below), we restricted our analysis to trichomes visible in the "trichome initiation zone" at the base of developing leaves. This region contains young trichomes that have not initiated secondary branching (Figure 2).

TRY Acts as a Negative Regulator of Overexpressed *GL1* and *R* Gene Activity

The *GL1* and *R* genes can be considered positive regulators, whereas *TRY* appears to act as a negative regulator of trichome development. To test whether *TRY* acts as a negative regulator of *GL1* and/or *R* gene function, we studied two *try* mutant lines overexpressing *GL1* or the *R* gene under the control of a strong constitutive cauliflower mosaic virus 35S promoter.

In 35S::*GL1* plants, virtually no trichome clusters were observed to be similar, as found in wild-type plants (Larkin et al., 1994) (Figure 3A and Table 1). By contrast, *try* 35S::*GL1* plants showed an enhanced phenotype (Figure 3B): the cluster frequency on *try* 35S::*GL1* leaves (13.9%) was found to be increased approximately twofold compared with the frequency on *try* mutants (8.3%) not overexpressing *GL1* (Table 1). This observation suggests that *TRY* limits the activity of overexpressed *GL1*.

For the analysis of *TRY* function in plants overexpressing the *R* gene, we used 35S::*R-GR*, an inducible form of the *R* gene with its N terminus fused to the vertebrate glucocorticoid receptor (Lloyd et al., 1994). Whereas *try* 35S::*R* mutants were difficult to grow, the inducible *try* 35S::*R-GR* line could easily be maintained under uninduced conditions. In our study, 35S::*R-GR* plants showed residual *R* gene activity

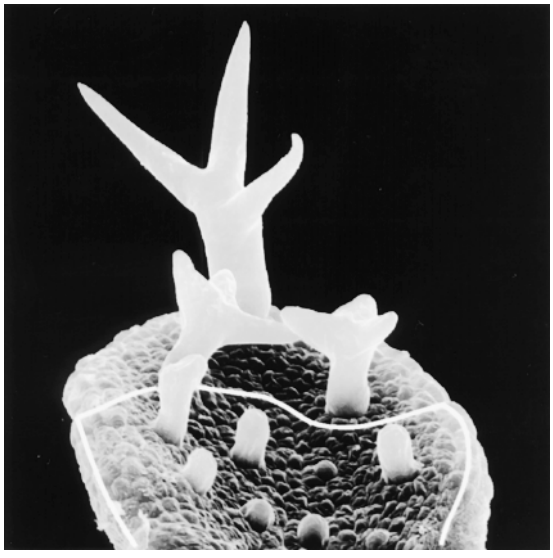

Figure 2. Wild-Type Trichome Initiation on a Young Leaf.

Scanning electron microscopy of a young leaf shows trichomes at different developmental stages. Trichome initiation is restricted to the trichome initiation zone, the border of which is marked by a white line. Trichomes become further separated due to cell divisions of intervening epidermal cells in more distal parts of the leaf (Hülskamp et al., 1999).

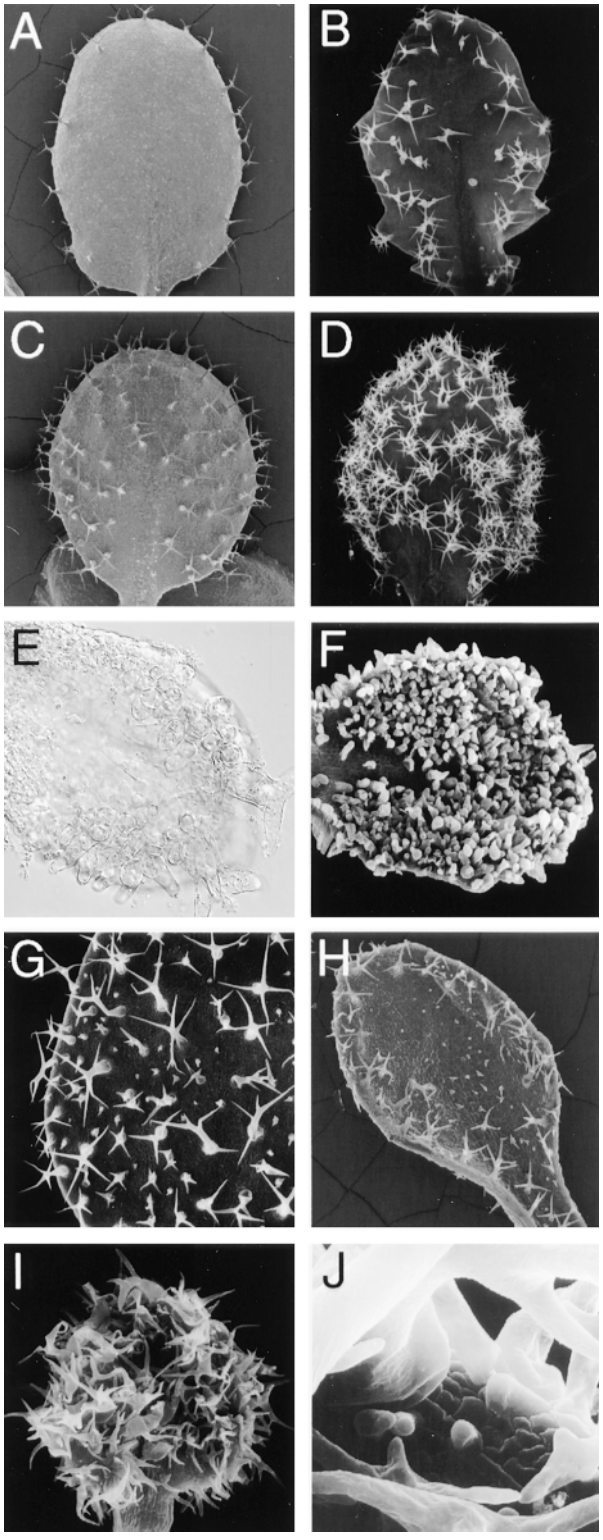

Figure 3. Cluster Formation in Different Genetic Combinations of 35S::*GL1*, 35S::*R-GR*, and *try* Mutants.

Scanning electron microscopy (**[A]** to **[D]** and **[F]** to **[J]**) and light microscopy **(E)** show the different mutant phenotypes.
(A) Overview of a 35S::*GL1* leaf.
(B) Overview of a *try* 35S::*GL1* leaf.
(C) Overview of an uninduced 35S::*R-GR* leaf.
(D) Overview of an uninduced *try* 35S::*R-GR* leaf.
(E) Young induced *try* 35S::*R-GR* leaf.
(F) Young *try* 35S::*R* leaf.
(G) Uninduced 35S::*R* leaf.
(H) Uninduced 35S::*GL1* 35S::*R-GR* leaf.
(I) 35S::*GL1* 35S::*R* leaf.
(J) High-magnification view of a 35S::*GL1* 35S::*R* leaf. Note that trichomes are still separated by intervening epidermal cells.

under uninduced conditions, because *ttg* mutants are partially rescued by uninduced 35S::*R-GR* (data not shown). 35S::*R-GR* plants have a cluster frequency of 0.4% (uninduced) and 7.3% (induced), respectively (Table 1). Compared with *try* or 35S::*R-GR*, uninduced *try* 35S::*R-GR* plants showed a significant increase in cluster frequency (31.5%), probably due to the residual activity of *R-GR* (cf. Figures 3C and 3D; Table 1). Similar to *try* 35S::*R* plants containing the uninducible *R* gene, induced *try* 35S::*R-GR* plants exhibited a strong increase in trichome density (Figures 3E and 3F). The cluster frequency and cluster size were determined in cleared whole-mount preparations of *try* 35S::*R-GR* leaves (Figure 3E). In this line, 83% of all trichome initiation sites contained two or more trichomes (Table 1). In addition, in induced *try* 35S::*R-GR* plants, the average number of trichomes per trichome initiation site was greatly increased. Induced *try* 35S::*R-GR* plants produced a significant number of larger clusters, with some having up to 40 trichomes (Table 2).

Overexpression of the *GL1* and *R* Genes in the Absence of *TRY*: Saturation of the System?

If *GL1*, *TTG*, and *TRY* are sufficient for trichome patterning, then overexpression of the *GL1* and *R* genes in the absence of *TRY* should result in the transformation of all epidermal cells into trichome cells.

Rosette leaves of Arabidopsis plants overexpressing *GL1* and *R* genes appear densely covered with branched trichomes; however, the leaves do not expand, making it difficult to assess the actual trichome pattern (Figure 3I) (Larkin et al., 1994). Mature 35S::*GL1* 35S::*R* leaves exhibit an increased number of trichomes when compared with the single lines (cf. Figures 3A, 3G, and 3I). Trichomes at the leaf margin were usually clustered, whereas trichomes on the leaf blade still showed a spacing pattern, with individual trichomes or trichome clusters separated by several epidermal

Table 1. Cluster Frequency in *try*, *gl1*, *ttg*, 35S::*GL1*, 35S::*R-GR*, and the Respective Double Mutant Combinations

Genotype	Cluster Frequency (%)[a]	No. of Trichome Initiation Sites
Landsberg *erecta*	{0.0 < 0.2 < 0.4}	1637[b]
try/+	{0.4 < 0.9 < 1.4}	1504[b]
try/*try*	{5.9 < 8.3 < 10.7}	528
ttg-1/+	{0.0 < 0.7 < 1.6}	295
ttg-w/+	{0.2 < 1.6 < 3.0}	317
ttg-w/*ttg-w*	{20.3 < 25.2 < 30.1}	314
ttg-10/+	{0.0 < 0.9 < 1.9}	328
ttg-10/*ttg-10*	{24.7 < 29.8 < 34.9}	308
gl1-EM1/+	0	302
gl1-EM2/+	{0.0 < 0.5 < 1.0}	772[b]
gl1-EM2/*gl1-EM2*	{0.0 < 0.3 < 0.9}	324
gl1-EM3/+	{0.0 < 0.1 < 0.3}	853[b]
gl1-EM3/*gl1-EM3*	0	53[c]
35S::*R-GR*/35S::*R-GR* NI[d]	{0.0 < 0.4 < 0.9}	542[b]
35S::*R-GR*/35S::*R-GR* I[e]	{4.5 < 7.3 < 10.1}	330
35S::*GL1*/35S::*GL1*	0	531[b]
try/*try* 35S::*R-GR*/35S::*R-GR* NI	{25.0 < 31.5 < 38.0}	197
try/*try* 35S::*R-GR*/35S::*R-GR* I	{76.5 < 83.8 < 91.1}	99
try/+ 35S::*GL1*/+	{0.0 < 1.0 < 2.9}	103
try/*try* 35S::*GL1*/+	{8.4 < 13.5 < 18.6}	171
try/+ 35S::*GL1*/35S::*GL1*	{0.0 < 2.0 < 4.7}	102
try/*try* 35S::*GL1*/35S::*GL1*	{10.0 < 13.9 < 17.8}	302
try/+ *ttg-1*/+	{3.9 < 6.9 < 9.9}	274
try/*try*+ *ttg-1*/+	{8.9 < 12.0 < 15.1}	426
try/+ *ttg-w*/+	{0.6 < 2.3 < 4.0}	307
try/*try ttg-w*/+	{15.2 < 18.9 < 22.6}	419
try/*try ttg-w*/*ttg-w*	{16.7 < 23.7 < 30.7}	139
try/+ *ttg-10*/+	{2.5 < 3.7 < 4.9}	923[b]
try/*try*+ *ttg-10*/+	{4.9 < 9.3 < 13.7}	298
try/*try ttg-10*/*ttg-10*[f]	{9.3 < 15.1 < 20.9}	252
try/+ *gl1-EM1*/+	{0.0 < 0.5 < 1.0}	744[b]
try/+ *gl1-EM2*/+	{0.0 < 0.2 < 0.4}	1132[b]
try/+ *gl1-EM3*/+	{0.0 < 0.1 < 0.3}	890[b]
ttg-1/+ *gl1-EM1*/+	{0.0 < 0.1 < 0.3}	811[b]
ttg-1/+ *gl1-EM2*/+	{1.7 < 3.8 < 5.9}	316
ttg-1/+ *gl1-EM3*/+	{0.0 < 0.2 < 0.5}	801[b]
ttg-w/+ *gl1-EM1*/+	{0.0 < 0.2 < 0.5}	608[b]
ttg-w/+ *gl1-EM2*/+	{0.0 < 0.2 < 0.5}	947[b]
ttg-w/+ *gl1-EM3*/+	{0.0 < 0.2 < 0.5}	947[b]
ttg-10/+ *gl1-EM2*/+	{1.4 < 3.5 < 5.6}	287
ttg-10/+ *gl1-EM3*/+	{0.0 < 0.1 < 0.3}	903[b]
try/*try* 35S::*GL1*/35S::*GL1 ttg-1*/*ttg-1*	{6.9 < 11.9 < 16.9}	160

[a] The mean value and the left and right border of a 95% confidence interval are shown.

[b] If the cluster frequency was <1% after the first 100 trichome initiation sites, mature trichomes were also counted.

[c] *gl1-EM3* mutants have only a few trichomes on rosette leaves.

[d] NI, not induced with dexamethasone.

[e] I, induced with 10 μM dexamethasone.

[f] In addition, we observed cell clusters of unusually large cells resembling trichome precursor cells. Because these cells were not round, as expected for young trichome cells, these clusters were not included; hence, the cluster frequency could be underestimated.

cells (Figure 3J). Thus, overexpression of the *GL1* and *R* genes in the presence of *TRY* is not sufficient to trigger trichome fate in all epidermal cells.

To test whether *TRY* is required for trichome patterning in 35S::*GL1* 35S::*R* plants, we constructed a line overexpressing the *GL1* and *R* genes in a *try* mutant background. Because 35S::*GL1* 35S::*R* plants stop growing after the first two rosette leaves are formed, we used the inducible 35S::*R-GR* line for these experiments. Compared with uninduced *try* 35S::*R-GR* (Figure 3D) or 35S::*GL1* 35S::*R-GR* (Figure 3H) lines, uninduced *try* 35S::*GL1* 35S::*R-GR* plants exhibited a marked reduction in growth and produced fewer rosette leaves that never fully expanded and a reduced number of flowers (Figure 4A). In addition, uninduced *try* 35S::*GL1* 35S::*R-GR* plants exhibited a striking trichome phenotype. Similar to those in 35S::*GL1* 35S::*R* plants, ectopic trichomes formed on various plant organs, including the abaxial surfaces of the first rosette leaves, the stamen, the pistil, and even between papillar cells (Figures 4A and 4B). In addition, trichome density was greatly increased on all plant organs. Thus, uninduced *try* 35S::*GL1* 35S::*R-GR* plants show a phenotype similar to plants overexpressing the *GL1* and *R* genes at high levels. These findings indicate that at least part of the trichome patterning found in 35S::*GL1* 35S::*R* plants is mediated by *TRY*. However, trichome patterning is not completely abolished in these plants. In high magnifications of rosette leaves, most trichomes were grouped in large clusters, and a significant number of trichomes were still separated by intervening epidermal cells (Figure 4C).

To test whether a further increase of *R* gene activity would trigger the complete transformation of all epidermal cells into trichome cells, *try* 35S::*GL1* 35S::*R-GR* seeds were germinated on agar plates containing 10 μM dexamethasone. All seeds germinated but then stopped growing after the initiation of the first rosette leaves. The incipient rosette leaves of these seedlings showed trichomes at various stages. These trichomes were separated by intervening epidermal cells (Figure 4D). Thus, even induced *try* 35S::*GL1* 35S::*R-GR* triple mutants do not exhibit a complete transformation of all epidermal cells into trichome cells. This result suggests the existence of at least one additional gene with a partially redundant role in the inhibitory pathway of trichome formation.

Genetic Interactions between *TRY* and *TTG*

TTG function is required for two aspects of trichome patterning. *TTG* acts positively on trichome initiation, as indicated by the lack of trichomes in plants carrying strong *ttg* alleles. In addition, *TTG* plays a role in the lateral inhibition of neighboring cells, similar to the role postulated for the *TRY* gene. The latter role is suggested by trichome cluster formation in plants carrying weak *ttg* alleles. To test whether *TRY* and *TTG* act in the same pathway during lateral inhibition, we studied *try*/+ and *ttg*/+ double heterozygous plants. If *TRY* and *TTG* act in the same pathway, one might

Table 2. Trichome Cluster Size in *try* 35S::*R-GR*

	try/*try* 35S::*R-GR*/35S::*R-GR*	
Cluster Size	Not Induced (%)[a]	Induced (%)[b]
1	77.4	16.1
2	21.0	11.1
3	1.6	11.1
4	–[c]	6.1
5	–	7.1
6	–	5.1
7	–	9.1
8	–	3.0
9	–	3.0
10	–	2.0
11 to 20	–	14.0
21 to 30	–	5.0
31 to 40	–	1.0
>40	–	3.0

[a] A total of 197 trichome initiation sites were analyzed.
[b] Induced with 10 μM dexamethasone. A total of 99 trichome initiation sites were analyzed.
[c] Dash indicates zero percent.

expect that a reduction in the activity of both genes would be sufficient to cause a phenotypic effect. A similar strategy has been used to study the genetic interactions between *CLAVATA1* and *CLAVATA3* (Clark et al., 1995). Several double heterozygous combinations of *try* with strong or weak *ttg* alleles were analyzed (Table 1). All double heterozygous plants revealed a significant increase in cluster formation, suggesting that both genes act in the same pathway. Additional support for this idea comes from the finding that both the *try ttg-w* and the *try ttg-10* double mutants did not show an additional increase in cluster frequency or maximal cluster size compared with *ttg-w* and *ttg-10*, respectively.

We performed a similar analysis using genetic combinations of *try* and different *gl1* alleles. For this analysis, we used one new strong *gl1* allele, *gl1-EM1*, and two newly identified weak *gl1* alleles, *gl1-EM2* and *gl1-EM3*. The two weak *gl1* alleles are characterized by a reduced number of trichomes in the center of the leaf and do not show any trichome clusters. All double heterozygous combinations of *try* and all *gl1* alleles tested showed no increase in cluster formation (Table 1).

Genetic Interactions between *GL1* and *TTG*

The very similar trichome phenotype of *gl1* and *ttg* mutants suggests that both genes have a very similar function during trichome formation. To test the genetic interaction between *GL1* and *TTG*, we analyzed different double heterozygous combinations of weak and strong *gl1* and *ttg* alleles. No increase in cluster frequency was found in double heterozygous combinations of strong alleles (Table 1). However, double heterozygotes of weak or strong *ttg* alleles with one weak *gl1* allele, *gl1-EM2*, produced significantly more trichome clusters than did plants heterozygous for either single allele (Table 1). The formation of clustered trichomes was highly specific for the respective allelic combinations (Table 1). Double heterozygous combinations of the weak *gl1-EM2* allele showed clustered trichomes together with the strong *ttg-1* allele and the weak *ttg-10* allele but not with the weak *ttg-w* allele, whereas all combinations with the weak *gl1-EM3* allele showed no increase in cluster formation. These observations could reflect that trichome patterning is sensitive to changes in the relative activity of *GL1* and *TTG*. Alternatively, the results could be interpreted as allele-specific effects, which may point to a direct molecular interaction of *GL1* with *TTG*.

Overexpression of *GL1* in the Absence of *TRY* Rescues the Strong Trichome Phenotype of the *ttg-1* Mutant

It has been reported previously that the constitutive expression of *GL1* cannot bypass strong *ttg* mutations, indicating that *GL1* and *TTG* act at the same point in trichome development (Larkin et al., 1994). However, it is also conceivable

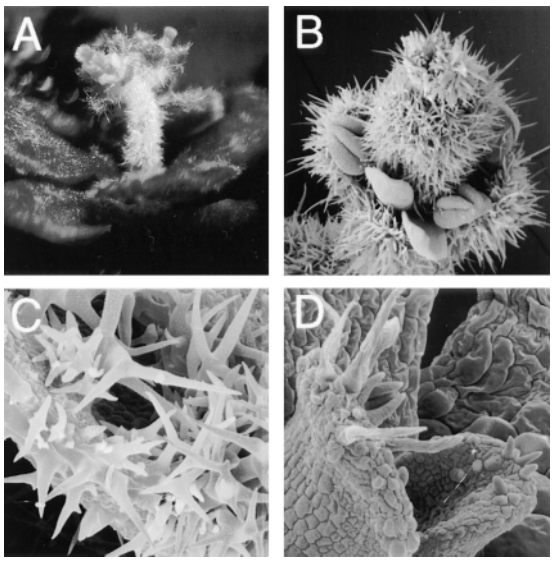

Figure 4. Phenotype of the *try* 35S::*GL1* 35S::*R-GR* Mutant.

Light microscopy **(A)** and scanning electron microscopy (**[B]** to **[D]**) were used to show the mutant phenotype.
(A) Overview of an uninduced *try* 35S::*GL1* 35S::*R-GR* plant.
(B) Flower of an uninduced *try* 35S::*GL1* 35S::*R-GR* plant.
(C) High-magnification view of an uninduced *try* 35S::*GL1* 35S::*R-GR* leaf. Note the intervening epidermal cells.
(D) Rosette leaves of an induced *try* 35S::*GL1* 35S::*R-GR* plant grown on dexamethasone-containing plates.

that in this rescue experiment the function of *GL1* is limited by *TRY*. To test this assumption, we constructed the *try* 35S::*GL1 ttg-1* triple mutant, with *ttg-1* being one of the strongest *ttg* alleles available. The triple mutant showed a rescue of the trichome phenotype of the *ttg-1* mutant with differentiated branched trichomes near the leaf margin and unexpanded *gl2*-like trichomes in the leaf blade (Figures 5C to 5F). The total number of trichomes was restored to that observed in *try* 35S::*GL1* mutants, although it was significantly reduced when compared with the wild type (Table 3). Also, the cluster frequency in the triple mutant was similar to that observed in *try* 35S::*GL1* plants (Table 1). A partial rescue of the trichome phenotype of the *ttg-1* mutant was also found in 35S::*GL1 ttg-1* double mutant plants (Table 3).

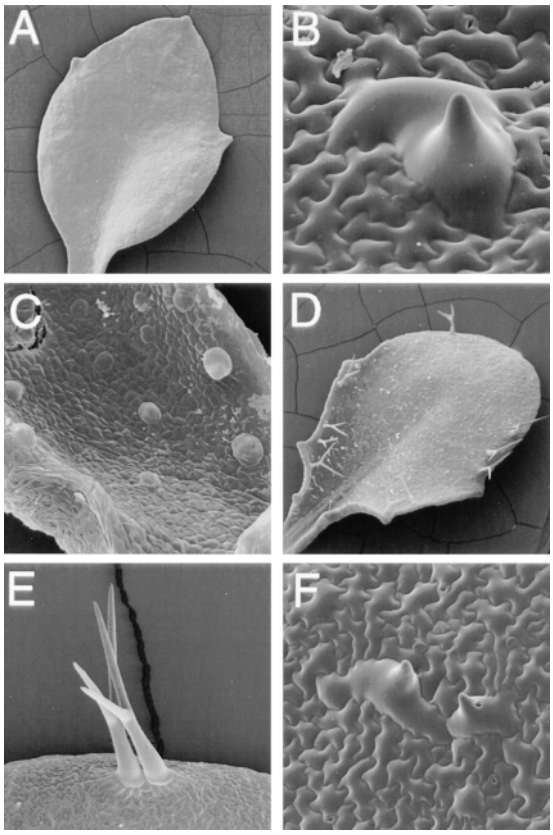

Figure 5. Rescue of the *ttg-1* Mutation.

Scanning electron microscopy was used to show the mutant phenotype.
(A) Overview of a 35S::*GL1 ttg-1* leaf.
(B) High-magnification view of a reduced 35S::*GL1 ttg-1* trichome.
(C) Overview of a young *try* 35S::*GL1 ttg-1* leaf.
(D) Overview of mature *try* 35S::*GL1 ttg-1* leaf.
(E) Differentiated *try* 35S::*GL1 ttg-1* trichome.
(F) High-magnification view of clustered *gl2*-like *try* 35S::*GL1 ttg-1* trichomes.

Table 3. Trichome Initiation Sites (TIS) in *try*, 35S::*GL1*, *ttg-1*, and the Respective Single and Double Mutant Combinations

Genotype	TIS per Leaf[a]	No. of TIS[b]
Columbia	42 ± 9	841
Landsberg *erecta*	17 ± 3	347
try/*try*	16 ± 4	319
ttg-1/*ttg-1*	0	0
35S::*GL1*/35S::*GL1*	9 ± 4	189
try/*try* 35S::*GL1*/35S::*GL1*	18 ± 10[c]	357
try/*try ttg-1*/*ttg-1*	0	0
ttg-1/+ 35S::*GL1*/+	35 ± 7	704
ttg-1/*ttg-1* 35S::*GL1* ND[d]	5 ± 4[e]	95
try/*try* 35S::*GL1*/35S::*GL1 ttg-1*/*ttg-1*	22 ± 4[f]	421

[a] Mean number of TIS ± SD.
[b] Trichomes were counted only on leaves 3 and 4. Leaf length was between 4 and 5 mm. Total number of leaves was 20.
[c] Sixty-four percent flat and reduced trichomes. Due to erupting subepidermal trichomes, the number of trichomes in the epidermis might be overestimeated.
[d] ND, not determined.
[e] Ninety-four percent flat and reduced trichomes.
[f] Fifty-three percent flat and reduced trichomes.

Whereas *ttg-1* mutants produced no trichomes on rosette leaves, the *ttg-1* 35S::*GL1* double mutant exhibited on average five trichomes per leaf. Most of these were not expanded (Figures 5A and 5B).

Maintenance of the Trichome Pattern

Initial trichome patterning results in the selection of single cells in a regular spacing pattern. How is this pattern fixed? Several double mutant combinations resulted in secondary trichome initiation immediately next to developing trichomes. Plants overexpressing the *R-GR* gene in the absence of *TRY* displayed a transformation of accessory cells into trichome cells under uninduced conditions (Figures 6C and 6D). Developing trichomes in the trichome initiation zone of the leaf were initially surrounded by a ring of apparently normal accessory cells. Later in leaf development, a variable number of accessory cells started to grow out, and most of them differentiated into mature trichomes (Figures 6C and 6D). This was observed for virtually all trichome initiation sites. A similar phenotype was observed for *try* 35S::*GL1* plants (cf. Figures 6A and 6B). Also, 35S::*GL1* 35S::*R-GR* plants under uninduced conditions exhibit this transformation of accessory cells into trichome cells. Thus, overexpression of both activators or overexpression of either of the activators in the absence of the inhibitor results in this phenotype. In summary, these data suggest that *TRY* is also involved in the lateral inhibition of trichome formation at later developmental stages.

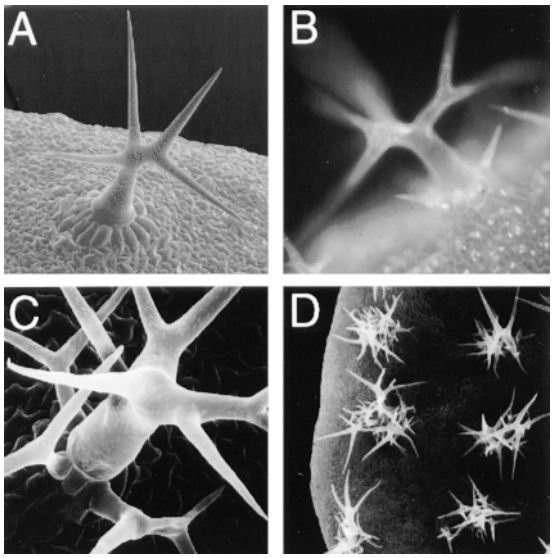

Figure 6. Transformation of Accessory Cells into Trichomes.

(A) Scanning electron microscopy of a single mature trichome in 35S::*GL1* with surrounding accessory cells.
(B) Whole-mount 4′,6-diamidino-2-phenylindole staining of a trichome cell in *try* 35S::*GL1*. Some of the adjacent accessory cells had initiated trichome differentiation.
(C) Scanning electron microscopy of a trichome cell with accessory trichomes in uninduced *try* 35S::*R-GR* plants.
(D) Overview of an uninduced *try* 35S::*R-GR* leaf.

DISCUSSION

Two lines of evidence suggest that trichome patterning in Arabidopsis does not involve cell lineage. The clonal analysis of wild-type patterning ruled out a cell lineage scenario in which the trichome cell is positioned in the center of its precursor cells (Larkin et al., 1996). The clonal analysis of trichome patterning in *try* mutants in this study shows that one of the key genes is not involved in cell lineage. Both experiments render it likely that the trichome spacing pattern is established by a competition mechanism that selects single trichome cells from otherwise equivalent epidermal cells.

Generation of a Spacing Pattern by Competition

Trichome initiation is confined to a relatively small region at the base of young leaves (Hülskamp et al., 1994; Larkin et al., 1996). At the time when developing trichomes first can be recognized, they are separated by approximately three or four epidermal cells (Hülskamp et al., 1994; Larkin et al., 1996). How is this spacing pattern generated? A simple scenario is that all cells within the trichome initiation zone are competent to become trichomes and compete with each other. The minimal requirements for this kind of cell-type selection have been formulated in a mathematical model (Meinhardt and Gierer, 1974). In this model, each cell produces an activator that stimulates its own production and that of an inhibitor. The activator acts locally and must have a self-enhancing property to allow the amplification of small stochastic local fluctuations. The inhibitor acts over long distances. A local increase of the activator would result in a locally increased production of the inhibitor, which in turn suppresses neighboring cells. According to this model, cluster formation would be expected either when the inhibitor levels are reduced or when the level of the activator is limited. Discussed below is a possible role for *GL1*, *TTG*/*R*, and *TRY* in the light of the predictions made by this model.

GL1 and *TTG*/*R* are considered to function as the activator and *TRY* to act as the inhibitor (Figures 7A and 7B). If *TRY* functions as the inhibitor, one would predict that *TRY* (1) would be able to negatively regulate *GL1* and/or *TTG*/*R* and (2) would be activated by *GL1* and/or *TTG*/*R*. In this study, we provide evidence for both predictions. A negative regulation of *GL1* and *TTG*/*R* by *TRY* is suggested by the finding that plants overexpressing *GL1* or the *R* gene show a strongly enhanced phenotype in the absence of *TRY*. This is most obvious in uninduced *try* 35S::*GL1* 35S::*R-GR* plants. Uninduced 35S::*GL1* 35S::*R-GR* plants show only little extra trichome formation, whereas the additional removal of *TRY* activity results in a strongly enhanced patterning phenotype.

The second prediction is that the function of *TRY* is coupled to the activity of the positive regulators. This prediction is supported by the increased cluster frequency in plants double heterozygous for *try* and different *ttg* alleles. Thus, a reduction of the inhibitory activity together with a reduction of the activating activity cause an enhanced phenotype. This finding establishes a link between the activating and the inhibitory pathways. Because this phenotypic enhancement was not observed in any of the double heterozygous combinations of *try* and *gl1* alleles, it is unlikely that cluster frequency in *try*/+ *ttg*/+ double heterozygous plants is simply due to a change in the relative dosage of the activator and inhibitor concentrations. Thus, it is conceivable that the function of *TRY* is regulated by the *TTG/R* pathway. It should be emphasized that the proposed inhibitory role of *TRY* is based solely on genetic arguments, and therefore, *TRY* is not necessarily required to act non–cell autonomously.

The proposed role of *GL1* and *TTG*/*R* is to promote trichome formation. But, how do these genes interact? We favor a model in which the *R* gene and *GL1* act at the same regulation level downstream of *TTG* to initiate trichome formation. This model accommodates several lines of genetic and molecular evidence. It has been shown previously that overexpression of the *R* gene cannot rescue *gl1* mutants (Larkin et al., 1994). This rules out a linear regulatory pathway and suggests that the *GL1* and *R* genes act at the same regulatory level. Recently, Szymanski and co-workers dem-

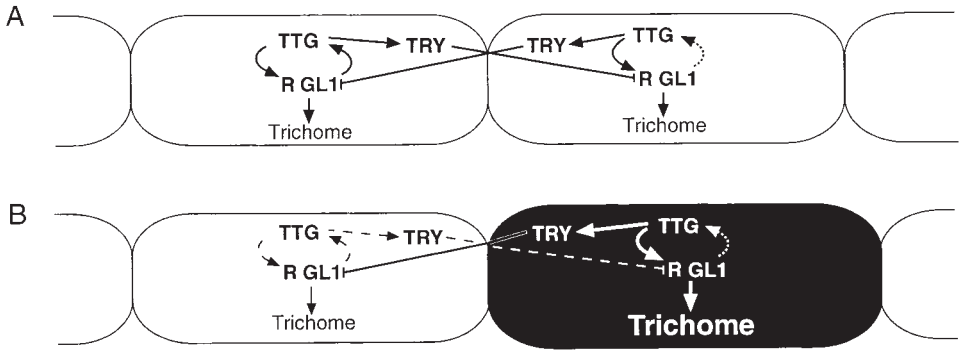

Figure 7. Tipping the Balance: A Genetic Model for Trichome Patterning in Arabidopsis.

(A) Two epidermal cells in a balanced situation. The *R* and *GL1* genes act together to promote trichome initiation, with *TTG* regulating the *R* gene. The double heterozygous combinations of *gl1* and *ttg* alleles suggest an interaction between *GL1* and *TTG*, probably with *GL1* functioning as a positive regulator of *TTG*. *TTG* and *TRY* act together in the inhibition of neighboring cells, with *TRY* acting as a repressor of trichome initiation.

(B) Two epidermal cells in a biased situation. The black cell has gained more competence, and as a result, this cell expresses more *GL1* and *R* and produces more *TRY* activity to inhibit trichome initiation in surrounding cells. The dashed line represents reduced interactions.

In **(A)** and **(B)**, arrows indicating activation of *TTG* by *GL1* are dotted to indicate that this interaction is postulated based on theoretical considerations and that evidence for this interaction is only circumstantial. Solid-line arrows indicate positive interactions.

onstrated a direct molecular interaction of *GL1* and the maize *R* gene (Szymanski et al., 1998a). The recent cloning of the *TTG* gene showed that *TTG* is not the *R* gene homolog (Walker et al., 1999). Therefore, it is likely that the *R* gene acts downstream of *TTG*, although the functional *R* gene homolog from Arabidopsis has not been identified.

That *TTG* acts upstream of *GL1* is suggested by our finding that the strong *ttg-1* phenotype is partially rescued by overexpression of *GL1* and even more restored in a *try* 35S::*GL1* background. However, according to the theoretical predictions, it is not possible that *TTG* acts simply upstream of *GL1*/*R* because no feedback loop would be established. A likely scenario is that *TTG* activity is dependent on *GL1* activity (Figure 7). This view is consistent with two observations. Plants overexpressing *GL1* produce a high number of trichome clusters when heterozygous for *TTG* (Larkin et al., 1994). A regulatory interaction between *GL1* and *TTG* is also suggested by the allele specificity of cluster formation in double heterozygous *gl1* and *ttg* mutants. Thus, in our model, *TTG* is not considered to be a general activator but to be involved in mediating the inhibitory signal and in the proposed positive feedback loop. In this scenario, the lack of trichome formation in *ttg* mutants would be due to the inability to locally enhance the activator concentration to levels high enough for trichome initiation. The phenotypic rescue of the *ttg* mutation in a *try* 35S::*GL1* background would be due to elevated levels of *GL1* that are sufficient for trichome initiation. Trichome spacing in the triple mutant would be mediated by the activity of other unidentified factors. The existence of additional factors is suggested by the finding that we were not able to transform all epidermal cells into trichome cells in *try* 35S::*GL1* 35S::*RGR* plants. A candidate for such a redundant gene is COTYLEDON TRICHOME1 (*COT1*), which has been identified as an enhancer of the *GL1* overexpression phenotype (Szymanski et al., 1998b).

Maintenance of the Trichome Pattern

When the leaf matures, developing trichomes become separated by cell divisions of intervening epidermal cells (Hülskamp et al., 1994). The initiation of new trichomes ceases in mature sections of the leaf (Hülskamp et al., 1994). It is likely that epidermal cells gradually lose the competence to become a trichome cell. This view is supported by the finding that *R* gene–induced trichome initiation is found only in young developing tissues but not in mature tissues (Lloyd et al., 1994). In contrast, overexpression of the *GL1* and *R* genes or overexpression of either one in the absence of *TRY* results in trichome initiation in more mature leaf areas. New trichomes, however, are initiated in immediate contact with already existing trichomes rather than between trichomes. This suggests that in plants overexpressing the *GL1* or *R* gene, cells immediately neighboring a trichome are competent to develop into trichomes and are subject to lateral inhibition by the central trichome cell. Because this late transformation of accessory cells into trichome cells was observed only in lines overexpressing positive regulators of trichome development, the extent to which lateral inhibition of neighboring cells is relevant in later stages of wild-type trichome development remains to be determined.

Multiple Roles of *GL1*, *TTG*/*R*, and *TRY* in Different Aspects of Trichome Development

Although the proposed role of *GL1*, *TTG*/*R*, and *TRY* in trichome patterning formally resembles well-characterized patterning processes in animals, the underlying molecular mechanisms are likely to be different. In animal development, pattern formation and cell fate determination are usually separate processes. A well-characterized example is the patterning of sensory bristles in the epidermis of Drosophila. A class of transcription factors, products of the *achaete-scute* gene complex, promote bristle development. Cellular interactions are mediated by a receptor–ligand—based mechanism that involves the *Delta* and *Notch* gene products (Campos-Ortega, 1993; Ghysen et al., 1993; Hinz et al., 1994; Artavanis et al., 1995). However, these genes appear to act exclusively in producing different cell fates within a given tissue, because the same set of genes acts not only in ectodermal patterning but also in endodermal patterning. The actual cell fate is determined by tissue-specific cues of the respective tissue layers (Yan and Jan, 1993; Tepass and Hartenstein, 1995).

By contrast, *GL1*, *TTG*/*R*, and *TRY* are involved not only in trichome patterning but also in the regulation of organ and tissue layer specificity of trichome formation (Hülskamp and Schnittger, 1998). Overexpression of the *R* gene triggers trichome initiation on organs that normally do not exhibit trichomes (Lloyd et al., 1992). Similarly, *try* 35S::*GL1* plants produce ectopic trichomes on different organs and in subepidermal tissue layers (Schnittger et al., 1998; Szymanski and Marks, 1998). This suggests that in trichome development, cell-type specification and patterning are mediated by the same set of gene functions. A possible entry point in cell differentiation is the regulation of endoreplication or DNA synthesis, which marks the transition from epidermal to trichome cell fate (Hülskamp et al., 1994). Interestingly, *GL1* and *TRY* are also involved in the regulation of endoreplication. *try* mutants show an increased DNA content compared with that of the wild type (Hülskamp et al., 1994). A role of *GL1* in the regulation of endoreplication is suggested by the finding that *try* 35S::*GL1* plants show an additional increase in nuclear DNA content (Schnittger et al., 1998; Szymanski and Marks, 1998). The dual role of *GL1* and *TRY* in trichome patterning and the regulation of endoreplication could reflect a functional link between the two processes.

TTG also appears to have a dual role in trichome patterning and differentiation. This is suggested by our finding that *try* 35S::*GL1 ttg-1* plants show a significant rescue of trichome initiation; however, trichome differentiation is still impaired in >50% of the developing trichomes. Strikingly, the phenotype is reminiscent of that of unexpanded trichomes in *gl2* mutants, suggesting that *TTG* is also required for the regulation of genes acting later in trichome morphogenesis.

In summary, *GL1*, *TTG*/*R*, and *TRY* are involved in multiple aspects of trichome development. It is likely that these different aspects reflect a complex molecular network underlying trichome patterning. Determining the roles of these genes in such a network is necessary for us to test our model of trichome patterning.

METHODS

Plants and Plant Culture

The *Arabidopsis thaliana* Landsberg *erecta* ecotype was used as a wild-type reference. The strong *ttg-1* allele was obtained from M. Koornneef (Agricultural University, Wageningen, The Netherlands). The weak *ttg-w* allele was obtained from G. Haughn (University of British Columbia, Vancouver, Canada), and the weak *ttg-10* allele was from D. Marks (University of Minnesota, St. Paul, MN). The *try-EM1* allele was isolated as a recessive mutation in a Landsberg *erecta* background (Hülskamp et al., 1994). The strong *gl1-EM1* allele and the two weak *gl1* alleles *gl1-EM2* and *gl1-EM3* were identified by screening M_1 progeny of ethyl methanesulfonate–mutagenized Landsberg *erecta* seeds. The 35S::*GL1* line (Larkin et al., 1994) and the pGGE4 line (Larkin et al., 1993) were obtained from D. Marks. The transgenic line containing the 35S::*R-GR* was obtained from A. Lloyd (Lloyd et al., 1994). Plants were grown under constant illumination, as described previously (Mayer et al., 1993).

The *try* 35S::*GL1* and *try* 35S::*R-GR* lines were constructed as described previously (Schnittger et al., 1998). The *try* 35S::*R* mutant was identified based on the new phenotype among the progeny of selfed F_1 plants from a cross between the homozygous 35S::*R* line and *try*. The *try*/*try* 35S::*GL1*/+ 35S::*R-GR*/+ plants were obtained from a cross between *try* 35S::*GL1* and *try* 35S::*R-GR*. The *try* 35S::*GL1 ttg-1* triple mutant was constructed by selecting plants from the F_2 progeny of a cross between *try* 35S::*GL1* and *ttg-1* displaying the phenotype of the *try* 35S::*GL1* mutant. The triple mutant was identified among the F_3 progeny of single plants exhibiting reduced trichomes and the transparent testa phenotype of the seeds. Homozygosity for *try* was confirmed by backcrosses with *try*. The presence of 35S::*GL1* was confirmed by kanamycin selection. The *ttg-1* 35S::*GL1* double mutant was identified among the F_2 progeny of a cross between 35S::*GL1* and *ttg-1* as glabrous, kanamycin-resistant plants. All double heterozygous combinations were F_1 plants of crosses between the respective parental lines.

For dexamethasone induction, 35S::*R-GR* plants were grown on soil, and whole pots with plants were immersed in 10 μM aqueous dexamethasone solution for 15 min. For in vitro germination under inductive conditions, seeds were grown on agar plates (0.5% agar, 0.5× Murashige and Skoog medium [Duchefa, The Netherlands], 3% sucrose, and 100 μM ampicillin) containing 10 μM dexamethasone.

Calculation of the Confidence Interval

The 95% confidence interval is defined as the interval that contains the mean value with a probability of 95%. The calculation of the confidence interval is based on the following formula (Kreyszig, 1979):

$$(n + c^2)p^2 - (2k + c^2)p + k^2/n = 0$$

where *n* is the number of trichome initiation sites. Trichome initiation sites are defined as leaf positions containing one trichome or a trichome cluster. The cluster frequency is *p*. The number of trichome

initiation sites containing more than one trichome is k. The chosen probability is c with $c = 1960$ at 95% probability (for details, see Kreyszig, 1979). Because $n - k$ was always large in this study, a simplified formula was used for the calculation of the confidence interval (Kreyszig, 1979): KONF $\{k/n - a = <p = <k/n + a\}$ with $a = c/n((k(n - k))/n)^{0.5}$.

Microscopy and Graphics Work

4′6-Diamidino-2-phenylindole staining was done as previously described (Hülskamp et al., 1994). For scanning electron microscopy, plant material was fixed in 100% methanol for 5 min, transferred to 100% ethanol, and further processed as previously described (Laux et al., 1996). In some cases, epoxy replicas of epidermal cells or immature trichome cells were used for scanning electron microscopic analysis (Williams and Green, 1988). Dental impression material (Colténe PRESIDENT light body; Coltène AG, Altstätten, Switzerland) was applied to the leaf surface for 5 min and peeled off. The negative molds were filled with Spurr's resin, polymerized at 70°C, and processed as described for fixed material.

Images were processed using Adobe Photoshop 3.0 (Adobe Systems Inc., San Jose, CA) and Aldus Freehand 7.0 (Aldus Corp., Seattle, WA) software.

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